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Associative Short-Term Synaptic Plasticity Mediated by Endocannabinoids

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Summary

Associative learning is important on rapid timescales, but no suitable form of short-term plasticity has been identified that is both associative and synapse specific. Here, we assess whether endocannabinoids can mediate such plasticity. In the cerebellum, bursts of parallel fiber (PF) activity evoke endocannabinoid release from Purkinje cell dendrites that results in retrograde synaptic inhibition lasting seconds. We find that the powerful climbing fiber (CF) to Purkinje cell synapse regulates this inhibition. Compared to PF stimulation alone, coactivation of PF and CF synapses greatly enhanced endocannabinoid-mediated inhibition of PF synapses. Retrograde inhibition was restricted to PFs activated within several hundred milliseconds of CF activation. This associative plasticity reflects two aspects of calcium-dependent endocannabinoid release. First, PF-mediated activation of metabotropic glutamate receptors locally reduced the dendritic calcium levels required for endocannabinoid release. Second, CF and PF coactivation evoked localized supralinear dendritic calcium signals. Thus, endocannabinoids mediate transient associative synaptic plasticity.

Introduction

Associative mechanisms of synaptic plasticity provide a powerful means of modifying the strength of synapses. In most cases, such mechanisms are regulated by the state of the postsynaptic cell, which is controlled by the interactions of synaptic inputs from multiple sources. For example, in the hippocampus, a subthreshold input can only induce LTP when it is coactivated with a strong input that fires the postsynaptic cell (Abbott and Nelson, 2000; Bi and Poo, 2001). Many synapse-specific forms of plasticity have been identified that contribute to long-lasting forms of associative learning (Bi and Poo, 2001) such as motor learning (Carey and Lisberger, 2002), fear conditioning (Marsicano et al., 2002), and spatial learning (Dragoi et al., 2003). In contrast, much less is known of transient forms of associative plasticity.

Numerous studies suggest that associative processes are important on rapid timescales (Brunel, 2003; Sandberg et al., 2003; Schultz and Dickinson, 2000). The ability of a neuron to integrate multiple inputs and use this information to rapidly modulate the strength of individual synapses during ongoing activity enables transient changes in a neural circuit during behavior without permanent modifications of the network. Such transient associative synaptic plasticity enables adaptation on rapid timescales during behavioral tasks for which long-term learning is not required (Schultz and Dickinson, 2000). For example, motor tasks such as limb movement or smooth pursuit eye tracking utilize error signals to correct errors and optimize performance (Kettner et al., 1997; Kitazawa et al., 1998). The use of error signals as feedback to correct and improve performance is effective as a general mechanism for corrective prediction in visual, reward, and motor systems (Widrow and Stearns, 1985). However, no physiological mechanisms have been described that allow synapse-specific associative short-term plasticity.

The endocannabinoid system is an attractive candidate to mediate short-term associative plasticity. At many synapses throughout the brain, endocannabinoids can be liberated from postsynaptic cells and bind to presynaptic CB1 receptors, thereby inhibiting synaptic transmission for tens of seconds (Alger, 2002; Freund et al., 2003; Wilson and Nicoll, 2002). Endocannabinoid release is controlled by both calcium-dependent (Brenowitz and Regehr, 2003; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) and calcium-independent (Kim et al., 2002; Maejima et al., 2001) mechanisms. The time course of this retrograde inhibition is suitable for providing feedback that could regulate network activity (Sandberg et al., 2003; Schultz and Dickinson, 2000), but it is not known if release of endocannabinoids is regulated in an associative manner.

Excitatory inputs to cerebellar Purkinje cells are well-suited to test for associative mechanisms of endocannabinoid release. Purkinje cells receive excitatory inputs from tens of thousands of weak granule cell parallel fiber (PF) synapses and from a single powerful climbing fiber (CF) synapse (Palay and Chan-Palay, 1974). Bursts of PF stimuli evoke localized release of endocannabinoids from Purkinje cell dendrites (Brown et al., 2003); however, the bursts required are more prolonged than those shown to occur in vivo during sensory stimulation (Chadderton et al., 2004). A possibility we test here is that the climbing fiber provides an associative signal to regulate endocannabinoid release from Purkinje cells in a manner similar to associative interaction of CF and PF synapses in long-term depression (LTD) of PF synapses (Ito, 2001; Wang et al., 2000).

Here, we show that activation of either PF synapses with a realistic burst or the CF synapse alone did not lead to retrograde inhibition of PF synapses. However, when activated together these two inputs led to strong transient inhibition of PF synapses lasting several seconds. This associative plasticity was mediated by calcium-dependent endocannabinoid release from the postsynaptic Purkinje cell and was restricted to PF synapses activated within several hundred milliseconds of CF activation. Retrograde inhibition was most prominent when CF activation followed PF activation. Two mechanisms underlie this associative plasticity. First, PF-evoked metabotropic glutamate receptor (mGluR1)

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activation lowered the calcium concentration required to evoke endocannabinoid release from Purkinje cells. Second, PF and CF coactivation led to large dendritic calcium signals. The transient feedback provided by associative retrograde inhibition could modulate cerebellar function on rapid timescales (milliseconds to seconds). Moreover, these experiments establish that endocannabinoid release can lead to associative short-term synaptic plasticity, and based on the wide distribution of CB1 receptors (Egertova et al., 2003; Pettit et al., 1998) this mechanism is likely to occur in many brain regions.

Results

Climbing Fiber Activation Enhances Endocannabinoid Release at PF Synapses

We tested the influence of CF activation on retrograde inhibition at PF synapses by measuring the effects of brief high-frequency conditioning trains of PF and CF stimuli on the amplitudes of PF excitatory postsynaptic potentials (EPSPs). We made whole-cell current-clamp recordings from Purkinje cells in sagittal cerebellar slices. Two extracellular electrodes were used to independently stimulate the CF and PFs (Figure 1A). Stimulus protocols consisted of low-frequency PF stimulation (0.5 Hz) that preceded and followed conditioning trains. Conditioning stimuli consisted of brief high-frequency trains delivered to the CF alone, the PFs alone, or both the CF and PFs. Representative experiments show the responses of a Purkinje cell to conditioning trains (Figures 1B–1E, left panel) and the effect of the conditioning train on the evoked EPSP (Figures 1B–1E, middle and right panels). Stimulation of the CF (five pulses at 100 Hz) evoked a series of complex spikes in the Purkinje cell but had no effect on the PF EPSP (Figure 1B). This shows that the CF alone could not evoke endocannabinoid release near PF synapses, despite strong Purkinje cell depolarization that produces widespread elevations of dendritic calcium (Lev-Ram et al., 1992).

We next tested whether CF activation could influence the retrograde inhibition evoked by PF activation. Rather than using a train of five to ten PF stimuli that is known to evoke endocannabinoid release and transient synaptic inhibition (Brown et al., 2003), we used shorter trains of just three pulses at 100 Hz that mimic in vivo granule cell responses to sensory stimuli (Chadderton et al., 2004; Eccles et al., 1966) and did not evoke substantial retrograde inhibition. PF activation alone caused the Purkinje cell to spike twice but did not depress PF EPSPs evoked after the train (Figure 1C). However, during trials where CF and PF activation were paired in rapid succession (Δt = 40 ms, measured from the center of each train), the PF EPSP was depressed by 82%, and this depression persisted for several seconds (Figure 1D). Bath application of the cannabinoid receptor antagonist AM251 (1 μM) eliminated the depression, indicating that it was mediated by endocannabinoid release that activated presynaptic CB1 receptors (Figure 1E).

Following PF conditioning trains of three to five stimuli, a CF train was able to reliably evoke strong retrograde inhibition at the PF synapse. The repeatability of the distinct effects of the different conditioning trains on the PF EPSP is illustrated by a plot of the EPSP following each conditioning train in this experiment (Figure 1F). Conditioning trains of the PF or CF alone or in combination were alternated at 2 min intervals. Whereas stimulation of the PF or CF alone did not evoke retrograde inhibition, paired stimulation consistently inhibited EPSPs by ~70%. Application of the CB1 receptor antagonist AM251 prevented inhibition of PF EPSPs, indicating that pairing the PF and CF stimulation inhibits PF synapses by evoking release of endocannabinoids. These results emphasize that realistic trains of PF activation bring the Purkinje cell near the activation threshold required for release of endocannabinoids, and subsequent stimulation of the CF synapse is sufficient to exceed this threshold and evoke endocannabinoid release.

In summary, we found that pairing CF activation with PF activation reliably induced associative short-term inhibition at PF synapses (Figure 1G). Conditioning stimulation of either the CF alone (3% ± 2% reduction) or PF alone (9% ± 3% enhancement) did not greatly alter PF responses, but when PF and CF synapses were coactivated, PF synapses were depressed by 62% ± 3% ($p < 0.0001$; $n = 16$). The CB1 receptor antagonist AM251 eliminated this associative depression. In AM251, the plasticity evoked by coactivation of PF and CF inputs was the same as that arising from PF stimulation alone (25% ± 8% and 29% ± 7% enhancement, respectively; $n = 6$). These experiments establish that pairing CF and PF stimulation leads to short-term associative plasticity mediated by endocannabinoids.

To further clarify the stimulus conditions required for this associative plasticity, we examined the effects of pairing CF activation with PF trains of different durations. This is shown for a representative cell (Figure 2A). For PF activation alone, there was a sharp threshold for inducing retrograde inhibition. Pairing CF and PF activation lowered the number of PF stimuli required to produce retrograde inhibition. Three PF stimuli alone resulted in 8% inhibition, but when paired with CF stimulation, the PF EPSP was inhibited by 58% (Figure 2A, upper panel). However, PF activation with ten stimuli produced near-maximal retrograde inhibition, so that CF coactivation caused little additional effect (Figure 2A, lower panel). In this example, CF activation reduced the number of PF stimuli required to obtain half-maximal inhibition from approximately seven to three (Figure 2B). This illustrates that associative short-term depression arising from PF and CF coactivation is most pronounced for granule cell bursts of two to five stimuli, which is typical of granule cell activity observed in vivo (Chadderton et al., 2004; Eccles et al., 1966).

We performed similar experiments in 12 cells and found that the CF had comparable effects on enhancing the extent of PF inhibition (Figures 2C and 2D). In different cells, the threshold for the number of PF stimuli that alone could induce retrograde inhibition varied from five to eight, and CF coactivation lowered this requirement by two to five stimuli. As a result, the onset of retrograde inhibition and the size of the CF effect were less pronounced in the average of many cells compared to that observed in individual cells (Figure

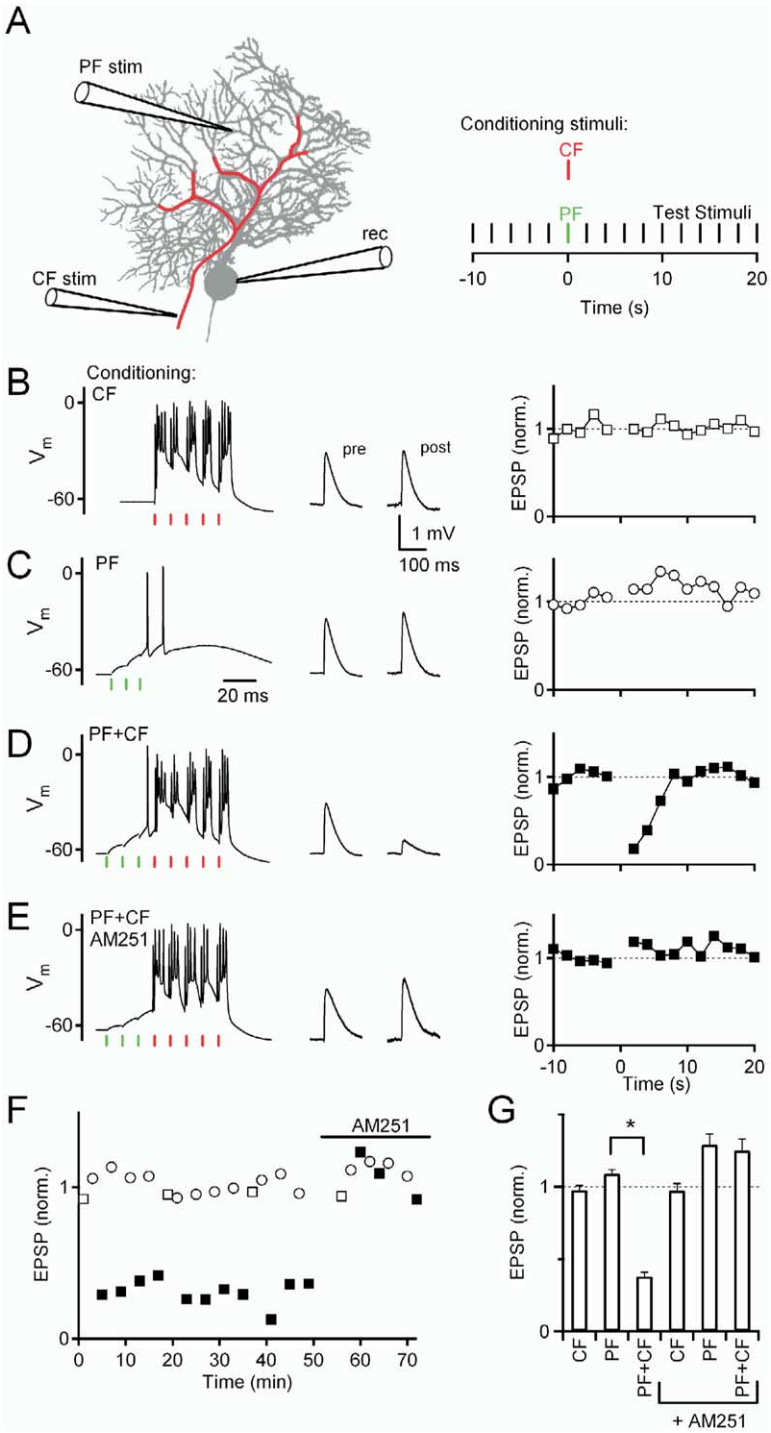

Figure 1. Pairing CF and PF Activation Produces Associative Short-Term Synaptic Plasticity

(A) (Left panel) Recording configuration and placement of stimulus electrodes. (Right panel) Illustration of conditioning stimulus protocols. Conditioning trains delivered to CF and PF synapses are indicated in red and green ticks, respectively.

(B–E) (Left panel) Responses of a Purkinje cell to conditioning trains. (Middle panel) EPSPs before (pre; average of five EPSPs) and after (post; average of two EPSPs) conditioning trains. (Right panel) EPSP amplitudes during 30 s trials, normalized to mean of first five EPSPs. Conditioning trains were delivered at time 0. Conditioning stimuli (100 Hz) in (B)–(D) were, respectively, five CF, three PF, and three PF + five CF stimuli (Δt = 40 ms). (E) Same conditioning stimulus as in (D) in the presence of the cannabinoid receptor antagonist AM251.

(F) Effects of conditioning stimuli (indicated by symbols in [B]–[E]) on the PF EPSP.

(G) Summary of effects of conditioning stimuli on PF EPSPs (control, n = 16; AM251, n = 6). Amplitudes of EPSPs following conditioning stimuli were normalized to preconditioning amplitudes. Asterisk indicates significant inhibition of PF EPSPs by paired CF + PF conditioning (Student's t test; $p < 0.01$).

2C). Overall, the effect of CF coactivation was to reduce the number of PF stimuli required for half-maximal retrograde inhibition roughly by a factor of two (Figure 2D). We found that retrograde inhibition and associative effects of the CF were eliminated when CB1 receptors were blocked with AM251 (Figures 2C and 2D). Moreover, retrograde inhibition was never observed in the presence of AM251, and instead there was transient enhancement that likely results from a buildup of presynaptic calcium (Zucker and Regehr, 2002). Thus, in control conditions retrograde inhibition is able to overcome this enhancement and leads to an overall depression in PF synaptic strength.

Postsynaptic Calcium Requirements for Associative Short-Term Inhibition

Previous studies have shown that both calcium-dependent and calcium-independent mechanisms can evoke

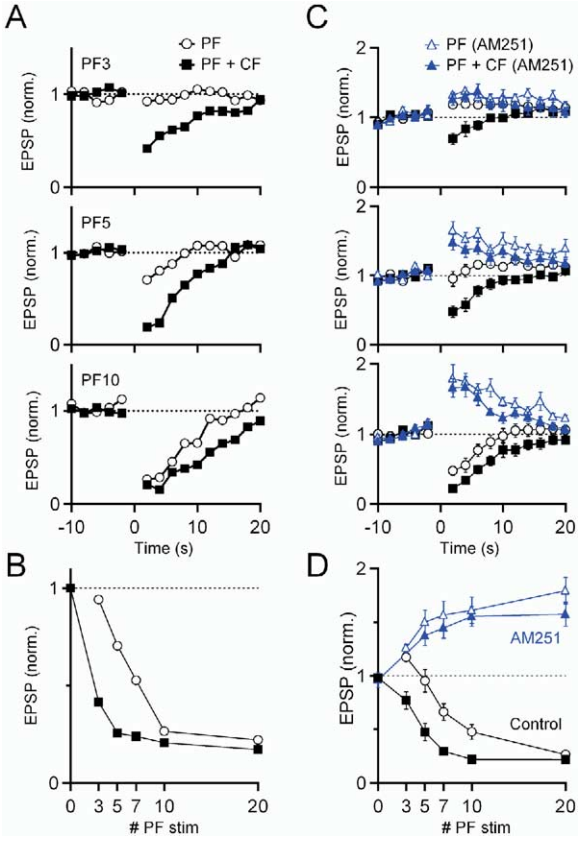

Figure 2. CF Activation Reduces the Number of PF Stimuli Required for Retrograde Inhibition

(A) Normalized EPSP amplitudes for a representative cell following 100 Hz conditioning trains consisting of three, five, and ten PF stimuli alone (open circles) or paired with five CF stimuli (Δt = 50 ms; filled squares).

(B) Inhibition of PF EPSP by PF or PF + CF conditioning trains. Data point for # PF stim = 0 refers to CF conditioning alone.

(C) Summary of the effects on the PF EPSP of conditioning trains consisting of three to ten PF stimuli alone (open circles) or with PF and CF conditioning (filled squares) for control conditions (black traces; n = 12) and in the presence of AM251 (blue traces; n = 6).

(D) Summary of EPSP inhibition.

endocannabinoid release from Purkinje cells (Kreitzer and Regehr, 2001; Maejima et al., 2001). We therefore included high concentrations of the calcium chelator BAPTA (20 mM) in the recording pipette to determine whether elevations of postsynaptic calcium contribute to retrograde inhibition or to the associative effects of the CF. We found that loading Purkinje cells with BAPTA prevented retrograde inhibition (Figures 3A and 3B). Similar results were obtained when cells were loaded with an internal solution containing BAPTA with added calcium to set resting calcium at ~60 nM (n = 5; see Experimental Procedures). This indicates that BAPTA eliminates retrograde inhibition by buffering stimulus-evoked calcium transients and not by affecting resting calcium levels. These experiments establish that elevations of postsynaptic calcium are essential for synaptically evoked retrograde inhibition by endocannabinoids and suggest that dendritic calcium could mediate CF effects on plasticity at PF synapses.

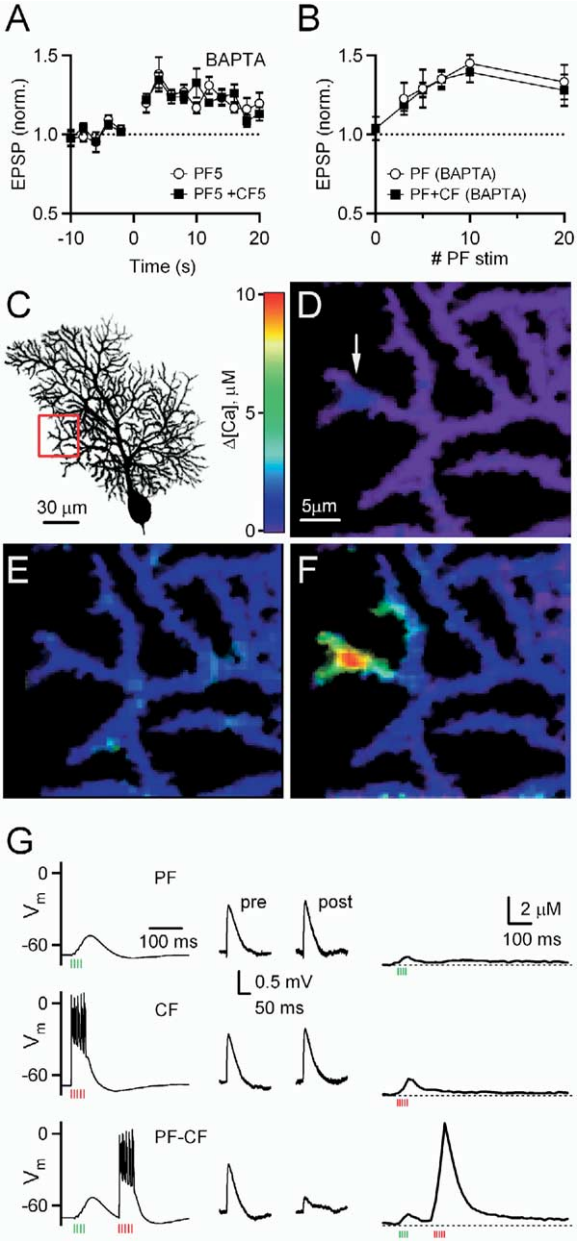

Figure 3. Postsynaptic Calcium and Associative Retrograde Inhibition

(A) BAPTA (20 mM) in the patch pipet prevented inhibition of EPSPs by 100 Hz conditioning trains of five PF or five PF plus five CF stimuli (n = 4).

(B) Normalized EPSP amplitudes following conditioning trains (n = 4) recorded from cells loaded with BAPTA.

(C–G) Calcium levels were measured with fura-FF, and retrograde inhibition was monitored simultaneously in the region indicated by the red box. Images of peak dendritic calcium levels are shown in response to four PF stimuli alone (D), five CF stimuli alone (E), and four PF + five CF stimuli with Δt = 150 ms (F). Placement of the PF stimulus electrode is indicated by an arrow in (D). (G) The corresponding electrophysiological response to stimulation (left panel), PF EPSPs (middle panel; average of two before and after conditioning train), and peak dendritic calcium transients recorded near the site of stimulation (right panel) are shown for the experiments depicted in (D)–(F). The timing of PF and CF stimuli are depicted by green and red ticks, respectively.

We therefore measured dendritic calcium levels produced by conditioning trains to assess the role of calcium in associative plasticity. We quantified dendritic calcium levels using fura-FF (K_D = 7.7 μM) as described previously (Brenowitz and Regehr, 2003; Brown et al., 2003) while simultaneously monitoring the electrophysiological response of the Purkinje cell, as shown for a representative experiment (Figures 3C–3G). Brief PF bursts of three to four stimuli that were below the threshold to evoke retrograde inhibition produced small localized calcium transients (0.6 ± 0.1 μM; range 0.3–1.1 μM; n = 10; Figures 3D and 3G). CF activation (five at 100 Hz) produced uniform dendritic calcium increases (1.2 ± 0.2 μM; range 0.6–2.0 μM; Figures 3E and 3G). However, CF trains delivered 150 ms after PF stimulation evoked large calcium increases (4.7 ± 0.9 μM; range 3–8 μM; Figures 3F and 3G) that were highly localized within the dendrite (width at half-maximum = 11.4 ± 0.7 μm; range 9–14 μm). These findings indicate that with stimulus conditions used in these experiments, neither PF nor CF stimulation alone elevates dendritic calcium levels sufficiently to trigger endocannabinoid release (Brenowitz and Regehr, 2003). The large supralinear enhancement of peak calcium evoked by pairing of PF and CF stimulation (Wang et al., 2000) suggests that enhanced dendritic calcium may contribute to the more pronounced retrograde inhibition following PF and CF coactivation.

Pathway Specificity of Associative Plasticity

Pairing CF and PF stimulation resulted in spatially restricted elevations of postsynaptic calcium, suggesting that retrograde inhibition of PF synapses by PF and CF coactivation is synapse specific. To test the synapse specificity of retrograde inhibition evoked by pairing PF (three to seven stimuli at 100 Hz) and CF (five stimuli at 100 Hz) conditioning trains, we used two stimulus electrodes to activate independent parallel fiber pathways separated by 18–28 μm (average = 23 μm; Figures 4A and 4B). EPSPs were monitored following conditioning trains delivered individually to each pathway (Figure 4C). When conditioning trains were delivered to PF pathway 1 and the CF synchronously, pathway 1 was inhibited but not pathway 2 (Figure 4C, upper panel). Similarly, when a conditioning train delivered to PF pathway 2 was synchronously paired with CF stimulation, PF pathway 2 was inhibited but not pathway 1 (Figure 4C, lower panel). Synapse specificity of retrograde inhibition was always seen following synchronous CF and PF coactivation. (Figure 4D; n = 5 cells, 10 pathways). We also found that retrograde inhibition was synapse specific when PF activation was followed 150 ms later by CF stimulation (Figure 4D), a particularly effective stimulus for evoking endocannabinoid release (see Figure 7).

Calcium Dependence of Associative Plasticity

To investigate mechanisms underlying the associativity of endocannabinoid release, we performed experiments in which we combined measurements of retrograde inhibition and dendritic calcium transients arising from PF and CF conditioning trains. Purkinje cells were loaded with fura-FF to determine dendritic calcium concentrations in these experiments.

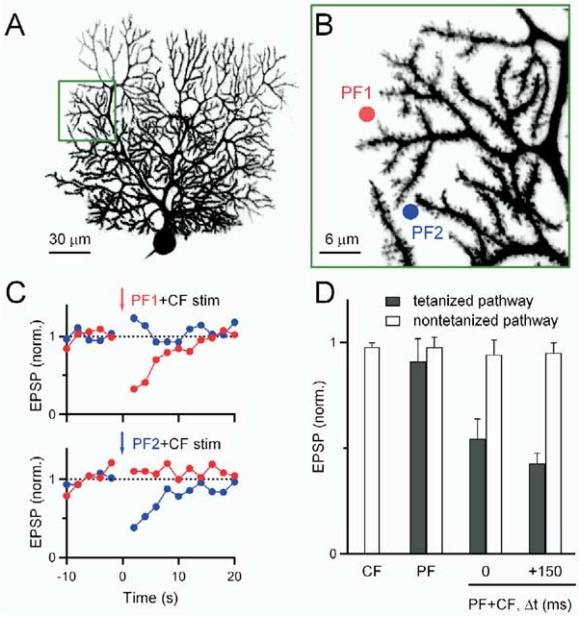

Figure 4. Pathway Specificity of Associative Retrograde Inhibition

(A and B) Two parallel fiber pathways (PF1 and PF2) were activated by stimulus electrodes positioned as indicated by red and blue circles.

(C) PF + CF tetani (Δt = +150 ms) delivered to pathway 1 (upper panel) and pathway 2 (lower panel) evoked retrograde inhibition only in the activated pathway.

(D) Summary data from five cells (ten pathways).

As shown in a representative experiment (Figure 5A), we reliably observed associative effects of CF and PF activation on both retrograde inhibition and on dendritic calcium levels. In this example, three PF stimuli produced a small increase in calcium, and no retrograde inhibition was observed (Figure 5A, upper panel). For five PF stimuli alone, the increase in calcium was 1.2 μM and consisted of a rapid increase followed by a slower component mediated by mGluR1 and IP_3-triggered release from internal stores (Finch and Augustine, 1998; Takechi et al., 1998). In this case, five PF stimuli alone were able to trigger the release of endocannabinoids and inhibit EPSPs by 36%. Pairing CF stimulation with five PF stimuli resulted in a much larger increase in calcium, and retrograde inhibition was 70% (Figure 5A, middle panel). The increase in calcium was larger than the summation of the calcium increases evoked by activation of the CF alone and the PF alone (Figure 5A, dashed lines). Following seven PF stimuli, the calcium increase was 4 μM for PF stimulation alone and 12 μM for PF and CF coactivation, and inhibition of EPSPs was near maximal (79% versus 81%, respectively; Figure 5A, lower panel).

The magnitude of the dendritic calcium signals and the resulting effect on synaptic strength is summarized for this cell for PF stimulation alone and for CF and PF coactivation (Figure 5B, open and closed circles, respectively). CF coactivation reliably produced a supralinear

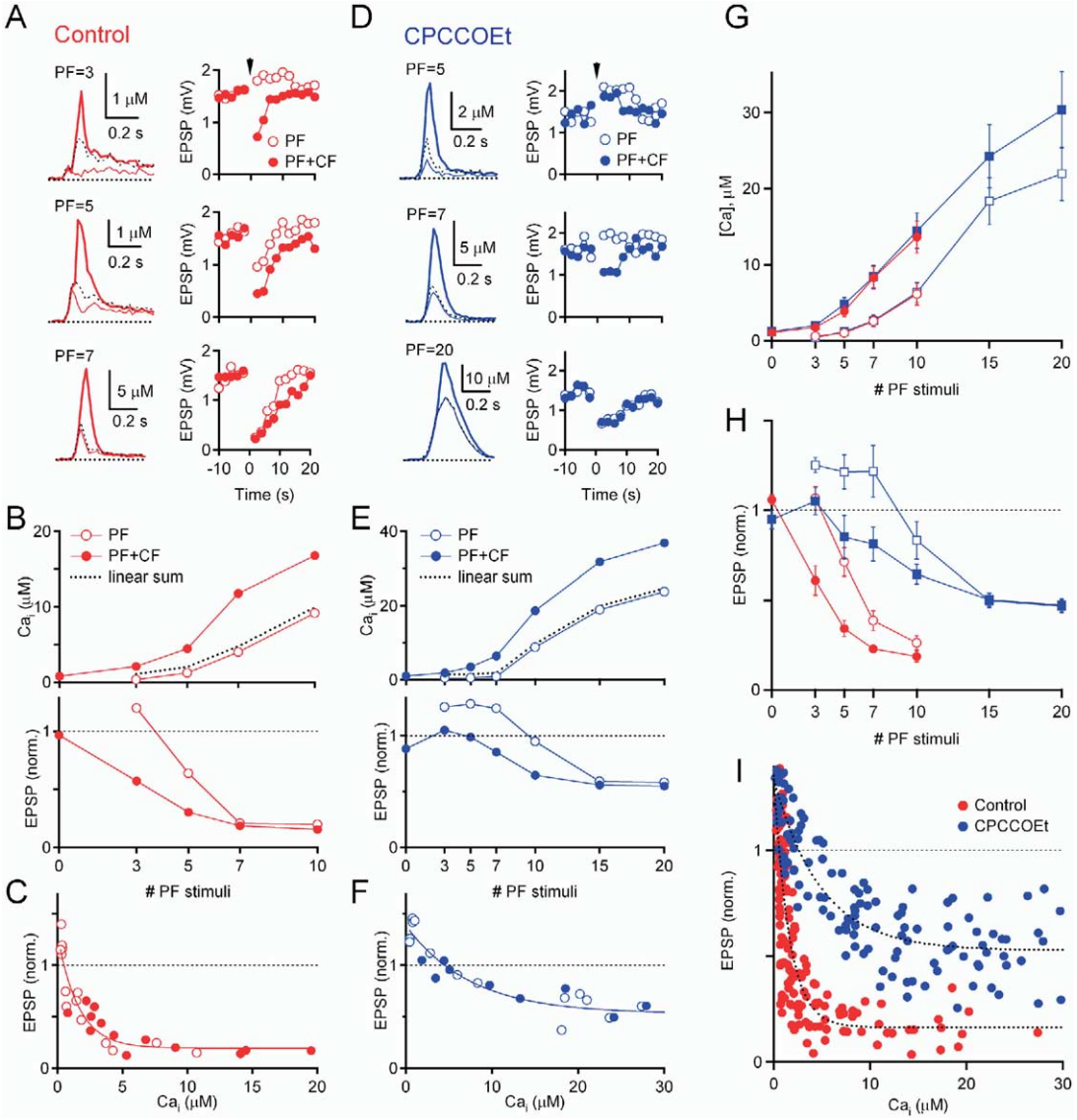

Figure 5. The Role of Dendritic Calcium and mGluR1 in Associative Retrograde Inhibition

PFs were activated alone (100 Hz trains) and accompanied by CF activation (five stimuli at 100 Hz; Δt = 75 ms). Dendritic calcium transients were measured with fura-FF, and the effects on EPSP amplitudes were quantified. Measurements were made both in control conditions (red) and in the presence of the mGluR1 antagonist CPCCOEt (100 μM; blue). A representative experiment is shown for control conditions in (A)–(C). (A) (Left panel) Dendritic calcium transients evoked by PF trains of three, five, and seven stimuli alone (thin traces) or accompanied by CF stimulation (thick traces). (Right panel) EPSP amplitudes before and after conditioning trains of PF alone (open symbols) or PF + CF (closed symbols) delivered at time 0 (arrow). (B) Peak dendritic calcium levels (upper panel) and normalized PF EPSP amplitudes (lower panel) in response to PF (open circles) or PF + CF (filled circles) conditioning trains. The dotted line indicates peak calcium predicted from linear summation of PF and CF responses. PF EPSP amplitudes determined from the average of two EPSPs measured at 2 and 4 s were normalized to preconditioning amplitudes. (C) Normalized EPSP amplitudes following conditioning trains plotted versus peak dendritic calcium levels. The data were approximated by an exponential fit (solid line) with half-maximal inhibition at 1.6 μM Ca and a maximum inhibition of 90%. A representative experiment in the presence of CPCCOEt is also shown in (D)–(F). (F) Normalized EPSP amplitudes following conditioning trains plotted versus peak dendritic calcium were approximated by an exponential decay (solid line) with half-maximal inhibition at 9.4 μM Ca and a maximal inhibition of 46%. (G) Summary of peak calcium levels and (H) normalized PF EPSP amplitudes in response to PF (open symbols) and PF + CF conditioning trains (filled symbols) under control conditions (n = 10; red traces) or in the presence of CPCCOEt (n = 8; blue traces). (I) Normalized PF EPSP amplitudes plotted versus peak Ca for all trials from all cells performed in control conditions (156 trials, 10 cells) and in CPCCOEt (152 trials, 8 cells). Data were approximated with exponential fits (see Results).

increase in dendritic calcium (Figure 5B, dashed line). It is likely that these supralinear calcium signals contribute to the associative release of endocannabinoids.

These experiments also provide insight into the calcium dependence of retrograde inhibition. The relationship between dendritic calcium and the EPSP magnitude reveals that substantial retrograde inhibition occurs even when dendritic calcium increases are <5 μM (Figure

5C). This is surprising in light of previous studies showing that half-maximal retrograde inhibition by endocannabinoids requires calcium increases of ~15 μM (Brenowitz and Regehr, 2003). While spatial gradients of calcium in the present experiments could complicate the determination of the precise calcium dependence of endocannabinoid release, it is clear that endocannabinoid release is far more calcium sensitive when triggered by synaptic inputs than when evoked by a postsynaptic depolarization.

The Role of mGluR1 in Endocannabinoid Release and Associative Plasticity

To investigate the heightened calcium sensitivity of endocannabinoid release, we focused on the role of mGluR1, which plays an important role in endocannabinoid release at the PF synapse (Brown et al., 2003). Fast synaptic transmission at PF synapses is mediated by AMPARs, and postsynaptic NMDARs are not present (Konnerth et al., 1990; Llano et al., 1991). In addition, mGluR1 is present on Purkinje cell dendrites and can be activated by stimulation of PFs with bursts (Batchelor et al., 1994; Tempia et al., 1998). mGluR1 can activate PLC and through DAG lipase lead to the synthesis of endocannabinoids (Alger, 2002; Freund et al., 2003; Piomelli, 2003). It has been shown that bath application of mGluR1 agonists can lead to the liberation of endocannabinoids (Galante and Diana, 2004; Maejima et al., 2001; Ohno-Shosaku et al., 2002; Varma et al., 2001), and it has been suggested that this can occur in a calcium-independent manner (Chevaleyre and Castillo, 2003; Kim et al., 2002; Maejima et al., 2001). Activation of mGluR1 triggers calcium release from internal stores that could contribute to endocannabinoid release. It has also been shown that synaptic activation of mGluR1 is sufficient to trigger endocannabinoid release when AMPARs are blocked (Brown et al., 2003; Ohno-Shosaku et al., 2002).

To examine the role of mGluR1, we repeated the systematic studies of retrograde inhibition and calcium measurements for CF and PF trains alone and together in the presence of the selective mGluR1 antagonist CPCCOEt (100 μM). A representative experiment is shown in Figures 5D–5F. CPCCOEt eliminated the late component of calcium that was mediated by release from internal calcium stores but did not have a significant effect on peak calcium levels. However, a pronounced effect on retrograde inhibition was observed. Five PF stimuli alone, which under control conditions resulted in a small amount of retrograde inhibition, did not produce significant retrograde inhibition in CPCCOEt (Figure 5D, upper panel). Pairing five PF stimuli with CF stimuli resulted in a calcium increase of 5 μM, but rather than inhibition, transient enhancement was observed. Seven PF stimuli alone evoked a calcium transient of 3 μM but did not result in retrograde inhibition. However, when paired with CF activation peak calcium reached ~11 μM, and significant retrograde inhibition was observed (Figure 5D, middle panel). This establishes that while mGluR1 activation has an important effect on synaptically activated endocannabinoid signaling, associative plasticity can occur even in the absence of mGluR1 activation. Following conditioning trains of 15 to 20 PF stimuli, both PF activation and PF plus CF activation produced comparable retrograde inhibition (Figure 5D, lower panel).

A summary of this representative experiment illustrates that peak calcium levels were unaffected by blocking mGluR1 and that the supralinear calcium increases were still present (Figure 5E, upper panel). There were, however, large effects of mGluR1 blockade on retrograde inhibition. In CPCCOEt, 15 to 20 PF stimuli were required to evoke prominent retrograde inhibition, whereas in control conditions only five stimuli were required. The associative effects of CF activation were still apparent, as indicated by the differences between the curves in Figure 5E (lower panel). In addition, the overall extent of inhibition in the presence of CPCCOEt was less pronounced than that in control conditions (inhibition of 50% compared to 90%). Perhaps the most striking observation is that dendritic calcium levels of more than 10 μM were required for maximal retrograde inhibition (Figure 5F).

A summary of the findings in control conditions and in the presence of CPCCOEt revealed a consistent and important role of mGluR1 activation by PF stimulation (Figures 5G–5I). Exponential fits used to approximate the data obtained in control conditions ($n = 10$) and in CPCCOEt ($n = 8$; Figure 5I, dotted lines) were significantly different (F test; $p < 0.01$; Glanz, 2002). Calcium required for half-maximal inhibition increased from 1.6 μM in control conditions to 6.5 μM in CPCCOEt ($p < 0.01$; Student's t test). Also, there was a significant difference in the maximal extent of retrograde inhibition, which was reduced from 84% ± 4% in control conditions to 47% ± 3% in CPCCOEt (Student's t test; $p < 0.01$). This suggests that in control conditions, activation of mGluR1 by PF conditioning stimuli plays an important role in making endocannabinoid release more sensitive to calcium. Thus, the ability of mGluR1 activation to lower the calcium requirement for endocannabinoid release contributes to enhanced retrograde inhibition evoked by associative activity of PF and CF synapses.

The Role of Internal Stores in Endocannabinoid Release and Associative Plasticity

Activation of mGluR1 could potentially influence endocannabinoid release in multiple ways. One possibility is the mGluR1 activation makes the biosynthetic pathway involved in endocannabinoid synthesis more sensitive to elevations of dendritic calcium. A second possibility is that calcium released from internal stores by mGluR1 activation could produce a local elevation of calcium that drives endocannabinoid release. Although peak calcium signals are not affected in the presence of CPCCOEt, delayed release of calcium from release from internal stores could contribute to endocannabinoid release. Also, a calcium-sensitive molecule involved in endocannabinoid production could be in close proximity to the IP_3-gated channels and therefore sensitive to a highly localized calcium signal that is difficult to detect with fluorescence techniques. We performed experiments to distinguish between these two possibilities. We used cyclopiazonic acid (CPA), which blocks the endoplasmic reticulum calcium-ATPase and de-

pletes internal calcium stores, to examine the role of calcium stores in endocannabinoid release. In these experiments, we used fura-5F to measure calcium because it has a higher sensitivity to calcium compared to fura-FF (K_D = 0.4 μM versus 7.7 μM) and is therefore better suited to measuring the calcium signals evoked by small numbers of PF stimuli.

We confirmed that CPA eliminated the late component of synaptically evoked calcium signaling that was apparent in control conditions, indicating that internal calcium stores were depleted. This is most clearly seen by comparing the calcium transients evoked by three PF stimuli in control conditions and in the presence of CPA (Figures 6A and 6B). However, peak calcium levels and supralinear increases in dendritic calcium were unaffected by depletion of internal calcium stores. This is apparent both in the representative experiments of Figures 6A and 6B and in the summary of the experiments provided in Figure 6C. In addition, retrograde inhibition was very similar in control conditions and in the presence of CPA (Figure 6D). Finally, there was no apparent shift in the calcium sensitivity of endocannabinoid release by depletion of internal stores (Figure 6E). Exponential fits used to approximate the data indicated that half-maximal inhibition occurred with peak dendritic calcium levels of 0.9 μM both under control conditions and in CPA, and the maximal inhibition was 69% in control conditions and 70% in CPA. Estimates of peak calcium required for half-maximal inhibition of PF EPSPs measured with fura-FF and fura-5F differed slightly (1.6 μM versus 0.9 μM). However, because of its higher affinity, fura-5F may provide more accurate measurements of calcium in this concentration range. In additional experiments using thapsigargin (10 μM) to deplete internal calcium stores, similar results were obtained (n = 4; data not shown).

These results indicate that for our experimental conditions release of calcium from internal stores does not contribute to retrograde signaling by endocannabinoids. Moreover, the associative effects of CF activation do not involve calcium release from internal stores. This suggests that mGluR1 activation participates in associative endocannabinoid release by making the production of endocannabinoids more sensitive to calcium (see Discussion).

The Timing Dependence of Associative Plasticity

An important aspect of associative plasticity is the timing requirement for associative inputs required to induce synaptic plasticity. Some types of long-term associative plasticity have strict timing requirements, while others do not require precise timing of the two associative signals (Abbott and Nelson, 2000; Ito, 2001). We determined the timing requirement of short-term associative plasticity mediated by PF and CF pairing and determined if supralinear increases in dendritic calcium could account for the associative effects of retrograde signaling by endocannabinoids. In these experiments, we paired PF and CF inputs and measured both retrograde inhibition and dendritic calcium levels. The relative timing of the inputs ranged from the CF stimulation preceding PF stimulation by 500 ms (Δt = –500 ms) to coincident activation (Δt = 0 ms) to PF activation preceding CF by 500 ms (Δt = 500 ms).

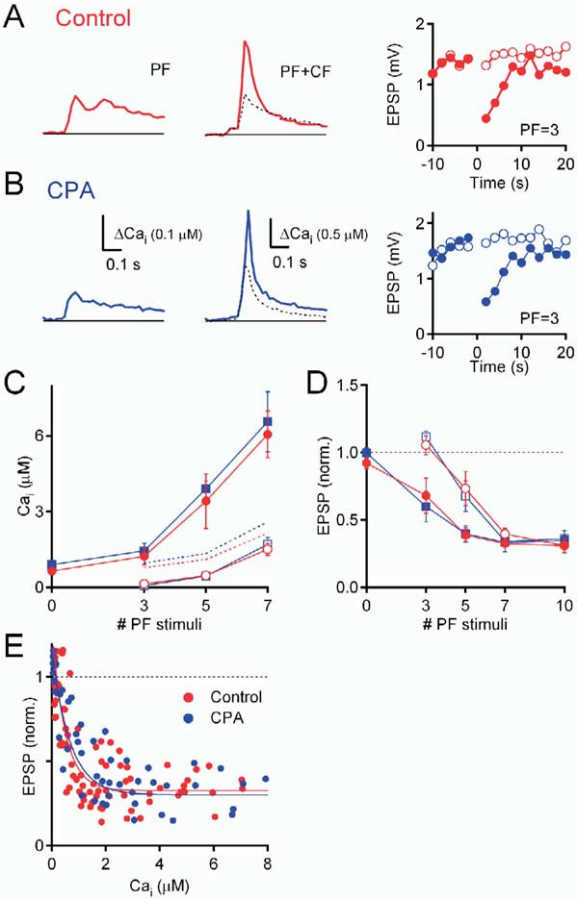

Figure 6. The Role of Internal Calcium Stores in Associative Retrograde Inhibition

Experiments were performed in control conditions (red) and in the presence of CPA (30 μM; blue) to deplete internal calcium stores. PFs were activated alone (100 Hz trains) and accompanied by CF activation (five at 100 Hz; Δt = 75 ms). Dendritic calcium levels were measured with fura-5F. (A and B) Representative experiments under control conditions and in CPA in which calcium transients were evoked by three PF stimuli alone (left panel) and by coactivation of PF and CF synapses (middle panel). The linear summation of the PF and CF responses is indicated by the dotted line. For both control conditions and in CPA, PF + CF pairing evoked inhibition of PF EPSPs (right panel, filled circles), whereas PF activation alone (right panel, open circles) did not. (C and D) Summary of peak calcium levels and normalized PF EPSP amplitudes in response to PF (open circles) and PF + CF conditioning trains (filled circles) under control conditions (n = 6; red traces) or in the presence of CPA (n = 4; blue traces). (E) Normalized EPSP amplitudes plotted versus peak dendritic calcium for all trials under control conditions (64 trials, 6 cells) and in CPA (42 trials, 4 cells). Data from control experiments and in CPA were approximated with exponential fits (see Results).

In a representative experiment, dendritic calcium transients are shown in response to three PF stimuli and five CF stimuli at the indicated times (Figure 7A, left panel). PF activation alone resulted in a rapid calcium transient followed about 200 ms later by a slow calcium increase arising from mGluR1 activation. CF activation alone produced a calcium increase of about 1 μM. The measured calcium transients (solid lines) arising from

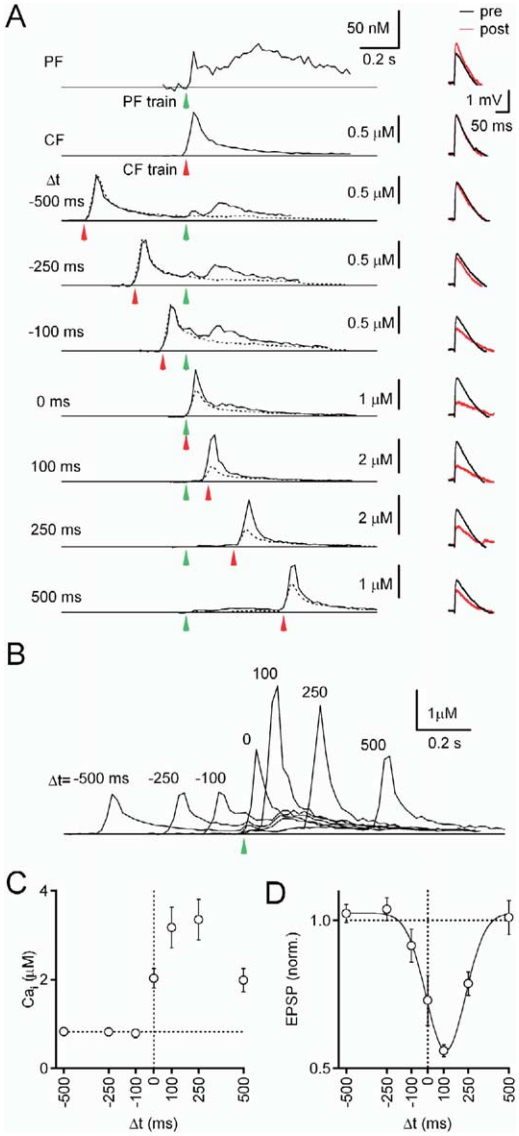

Figure 7. Timing Dependence of Calcium Supralinearity and Associative Short-Term Inhibition

Calcium transients were measured with fura-5F, and retrograde inhibition was monitored in response to PF stimulation (three at 100 Hz), CF stimulation (five at 100 Hz), and paired PF and CF stimulation with separation of indicated values of Δt. (A) Dendritic calcium transients ([A], left panel) and EPSPs ([A], right panel) are shown for PF stimulation alone, CF stimulation alone, and both PF and CF activation in a representative experiment. PF trains are indicated by green arrows, and CF trains are indicated by red arrows. For each pairing, the linear sum of the PF and CF calcium transients is indicated by a dotted line. Ordinates are scaled to the peaks of each trial. (Right panel) Average of five PF EPSPs preceding (black traces) and the average of two PF EPSPs at 2 and 4 s (red traces) following conditioning stimuli. (B) Calcium transients from (A) evoked by PF and CF pairing with indicated values of Δt are plotted on the same scale to illustrate the differences in peak calcium transients. Traces are aligned relative to the timing of the PF train (green arrow). (C and D) Summary of peak calcium levels and EPSP amplitudes in response to paired stimulation of PF and CF separated by time Δt (n = 4).

PF and CF coactivation are compared to the arithmetic sum of the responses to CF activation alone and PF activation alone (dashed lines). To emphasize the supralinear component of the calcium transient, traces were scaled to the peak of each trial in Figure 7A. When CF stimulation came first, PF activation triggered a large delayed response reflecting release from internal stores (Figure 7A; Δt = –500 ms to –100 ms). When PF activation and CF activation were synchronous (Δt = 0 ms), or when PF activation preceded CF activation (Δt = 100 ms to 500 ms), the peak calcium increase in response to the CF conditioning train was much larger than that evoked by the CF alone. CF activation evoked the largest increase in calcium for Δt = 100 ms. We also found that CF and PF stimulation evoked pronounced retrograde inhibition, as seen by comparing the control EPSPs (Figure 7A, right panel, black traces) to the EPSPs following paired CF and PF activation (Figure 7A, right panel, red traces). The dependence of peak calcium increases on the timing of PF and CF stimulation is most clearly seen by plotting the calcium transients from Figure 7A on the same graph, aligned to the time of the PF conditioning train (Figure 7B). Similar trends in the effect of timing on peak calcium transients were seen in four experiments in which dendritic calcium and retrograde inhibition were measured while varying the timing of PF and CF stimulation (Figures 7C and 7D).

Several conclusions can be drawn from these experiments. First, there is a relatively broad requirement for the timing of PF and CF activation, with greater inhibition occurring when PF stimulation preceded CF stimulation. For example, there was significantly more retrograde inhibition for Δt = +100 ms compared to Δt = –100 ms (44% versus 9%, respectively; n = 4; $p < 0.001$; paired Student's t test). The extent of depression was well approximated by a Gaussian fit with a width at half-maximal inhibition of 280 ms centered at Δt = +110 ms. Second, the supralinear increase in calcium observed for PF stimulation following CF stimulation and the peak calcium levels measured for these two times also differed significantly. There was a significantly larger increase in calcium for Δt = +100 ms compared to Δt = –100 ms (3.2 versus 0.8 μM, respectively; n = 4; $p < 0.01$; paired Student's t test). This suggests that the properties of calcium transients evoked by paired PF and CF stimulation likely contribute to the observed dependence of retrograde inhibition on the relative timing of PF and CF activation.

A comparison of the calcium signals and inhibition evoked by PF and CF stimulation also reveals that the supralinear effects of calcium are not sufficient to explain the dependence of retrograde inhibition on the timing of PF and CF activation. For example, the calcium transient observed for Δt = +500 ms is similar to that evoked by coincident activation of PF and CF stimulation (Δt = 0 ms), but synaptic inhibition is absent for Δt = 500 ms and prominent for Δt = 0. Therefore, activation of mGluR1 may also contribute to the timing dependence of associative plasticity by influencing endocannabinoid release and the extent of retrograde inhibition.

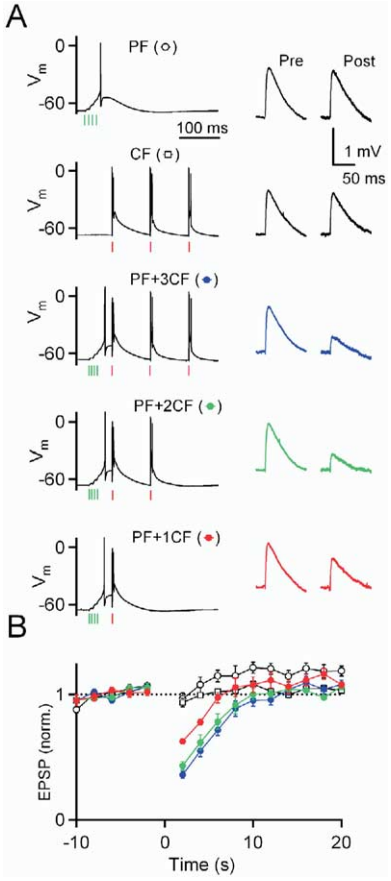

Figure 8. Sensitivity of Retrograde PF Inhibition to Physiological Patterns of CF Activation

(A) (Left panel) Response of a Purkinje cell to conditioning trains. (Right panel) EPSPs before (pre; average of five EPSPs) and after (post; average of two EPSPs) conditioning trains. Green ticks, PF stimuli. Red ticks, CF stimuli.

(B) Time course of EPSP amplitudes (normalized to average of five EPSPs before conditioning stimulus) during trials indicated by plot symbols and colors in (A).

Associative Short-Term Plasticity after Low-Frequency CF Activation

Thus far, we have focused on the effects of CF activation with five pulses at 100 Hz to produce associative short-term plasticity. We next tested the sensitivity of PF plasticity to patterns of CF activation similar to those observed in vivo (one to three stimuli at 10 Hz) (Armstrong and Rawson, 1979; Carey and Lisberger, 2002; Schwarz and Welsh, 2001). CF activation occurred after PF stimulation to maximize the extent of the inhibition. For PF activation that did not produce retrograde inhibition, we found that three or two CF stimuli at 10 Hz, and even a single activation of a CF, all produced retrograde inhibition (Figure 8A). Similar results were seen in six of six cells (Figure 8B). On average, a single CF stimulus following PF stimulation inhibited PF EPSPs by 38%. These results indicate that even a single CF-evoked complex spike can have powerful effects on retrograde inhibition of PF synapses and suggest that CF and PF coactivation likely control endocannabinoid-mediated inhibition of PF synapses under physiological conditions.

Discussion

Endocannabinoids Mediate Associative Short-Term Plasticity at Parallel Fiber Synapses

We identify a rapid form of associative short-term synaptic inhibition mediated by endocannabinoid release. This associative plasticity results from pairing PF and CF inputs. Inhibition is synapse specific and requires associative stimulation of CF and PF inputs within a temporal window of several hundred milliseconds. Substantial associative inhibition of PF EPSPs occurred following coactivation of PFs with a burst typical of that observed in vivo along with a single CF activation. This associative short-term plasticity results from the interaction of two mechanisms. First, PF-mediated mGluR1 activation enhances the calcium sensitivity of endocannabinoid release. Second, large supralinear elevations of calcium are locally evoked by paired activation of PF and CF inputs. These findings indicate that endocannabinoids likely provide a signal for short-term associative plasticity in vivo.

Activation of either CF or PF synapses with brief bursts was ineffective in evoking retrograde inhibition of PF synapses. Although high-frequency CF activation evoked widespread increases in dendritic calcium of 1–2 μM, it did not release endocannabinoids at PF synapses. The absence of PF inhibition in response to CF trains is consistent with the high postsynaptic calcium levels required for depolarization-evoked endocannabinoid release (Brenowitz and Regehr, 2003). When PF synapses were activated in isolation, we observed only a small degree of retrograde inhibition following activation of PF synapses with short bursts (<5 stimuli) of the sort observed in vivo (Chadderton et al., 2004). This suggests that even though longer bursts of PF activation alone can evoke endocannabinoid release (Brown et al., 2003), realistic bursts of PF activity alone produce only modest retrograde inhibition. Coactivation of the CF with realistic PF bursts, however, evokes much greater levels of retrograde inhibition. This suggests that associative CF and PF activity may be required for efficient release of endocannabinoids under realistic conditions.

The Role of Dendritic Calcium Transients and mGluR1 Activation in Associative Plasticity

Previous studies have shown that both calcium-dependent and calcium-independent mechanisms can evoke endocannabinoid release (Brenowitz and Regehr, 2003; Kim et al., 2002; Kreitzer and Regehr, 2001; Maejima et al., 2001), but their relative importance under physiological conditions was not known. Our observation that buffering postsynaptic calcium with BAPTA blocks synaptically evoked retrograde inhibition establishes that dendritic calcium increases are required for synaptically evoked endocannabinoid release at the PF synapse.

In our studies of associative short-term plasticity, CF activation leads to dendritic calcium increases that are restricted to the region of activated PF synapses (Figure 3). Moreover, nearby PF synapses that were not in

the region of supralinear calcium transients did not undergo retrograde inhibition (Figure 4). The large supralinear dendritic calcium elevations following coactivation of PF and CF synapses may thus contribute to associative short-term inhibition. PF and CF coactivation has been shown to evoke supralinear elevation of dendritic calcium (Wang et al., 2000). Under our experimental conditions, in which we locally activate a number of PF synapses, dendritic calcium supralinearity is not dependent on mGluR1 activation (Figure 5) or on release of calcium from internal stores (Figure 6). Supralinearity is likely to result from enhanced depolarization by coactivation of CF and PF synapses that could increase calcium influx. In addition, localized saturation of endogenous calcium buffers (Maeda et al., 1999) could contribute to calcium supralinearity. However, for sparse PF activation (Wang et al., 2000) it is possible that internal calcium stores could be important for endocannabinoid release. The temporal requirements for supralinearity were similar to those observed in this study and were well correlated with the temporal window for induction of PF LTD (Wang et al., 2000). Thus, mechanisms that allow supralinear elevation of calcium in response to coactivation of CF and PF synapses contribute to associative plasticity on both short and long timescales. Our studies point to the importance of supralinear calcium increases in associative release of endocannabinoids, but further studies will be needed to clarify the mechanism underlying this supralinearity.

mGluR1 also plays an important role in associative plasticity. In the presence of an mGluR1 antagonist, many more PF stimuli are necessary to evoke retrograde inhibition, whether delivered alone or paired with CF activation (Figure 5). Our results indicate that mGluR1 does not influence endocannabinoid release by triggering calcium release from internal stores (Figure 6). Our findings suggest that a primary role of mGluR1 is to lower the calcium requirement of endocannabinoid production. Molecular targets of mGluR1 activation and enzymes regulating endocannabinoid biosynthesis are sensitive to calcium levels (Bisogno et al., 2003; Hirose et al., 1999; Rebecchi and Pentyala, 2000; Stella et al., 1997). Moreover, the role of mGluR1 in enhancing the calcium sensitivity of endocannabinoid release has important implications for long-term forms of associative plasticity that involve retrograde signaling by endocannabinoids (Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Robbe et al., 2002; Sjostrom et al., 2003). Further studies will be required to clarify the mechanism responsible for the apparent reduction in the calcium requirement for endocannabinoid release mediated by mGluR1 activation. It will be of particular interest to determine if there is a shift in the calcium dependence of a single mechanism of endocannabinoid release, or whether there are distinct mechanisms that have different calcium dependencies.

Timing Dependence of Associative Short-Term Plasticity

Both the supralinear increases in calcium and the effect of mGluR1 on the calcium sensitivity of endocannabinoid release appear to shape the timing dependence of associative plasticity. PF stimulation leads to local mGluR1 activation, a local calcium increase, and a local decrease in the calcium levels required to evoke endocannabinoid release. For paired PF and CF activation, there is both a reduced calcium requirement and sufficient calcium to trigger endocannabinoid release. The supralinear increase in calcium is greatest when CF and PF fibers are activated synchronously, or when CF activation follows PF activation by up to 500 ms. This contributes to the broad timing of associative plasticity that is most prominent for PF activation followed by CF activation. But a comparison of the peak calcium levels and the extent of retrograde inhibition for $\Delta t = 0$ and $\Delta t = 500$ ms is revealing. Despite the fact that the peak calcium levels are similar, there is prominent inhibition for $\Delta t = 0$ but not for $\Delta t = 500$ ms. This establishes that factors other than dendritic calcium contribute to the timing requirement of retrograde inhibition. One possibility is that a transient reduction in the calcium dependence of endocannabinoid release by mGluR1 activation narrows the timing requirement for associative endocannabinoid release.

Implications for Cerebellar Function

The instructive role of the CF and the timing requirement for PF and CF coactivation are reminiscent of LTD (Ito, 2001; Wang et al., 2000). However, in contrast to the requirement of repeated CF and PF pairings for LTD induction, the plasticity we describe leads to PF depression after a single pairing. Inhibition of PF EPSPs lasts for several seconds and does not result in long-term changes in PF strength. Such plasticity may therefore play an important role in regulating PF strength on rapid timescales by dynamically modulating the ability of granule cells to regulate Purkinje cell firing during ongoing tasks for which no long-term learning is required.

Our findings suggest that an important way that CF activity can regulate the output of the cerebellum during ongoing activity is by providing rapid localized feedback to coactive PF synapses. Such feedback improves the performance of control systems (Schultz and Dickinson, 2000; Widrow and Stearns, 1985). This has been recognized in numerous models of cerebellar function (Churchland and Lisberger, 2001; Ito, 1984; Kawato and Gomi, 1992; Kettner et al., 2002).

Climbing fiber activity can encode both predictions and errors of arm movements during behavioral trials (Kitazawa et al., 1998). Thus, during motor activity, subsets of PFs that fire bursts within an appropriate time window relative to CF activity can undergo associative short-term inhibition. This modulation of PF strength could be important for fine motor control. Previous models of cerebellar function have emphasized that climbing fiber prediction errors provide feedback during predictive eye movements and are crucial for optimizing smooth pursuit eye tracking. CF input could be used to signal deviations from a predicted target motion and lead to dynamic changes in PF synapses to optimize performance (Kettner et al., 1997; Schultz and Dickinson, 2000). The mechanism of associative short-term plasticity presented here suggests that endocannabinoids could provide an associative signal to regulate cerebellar function during such tasks.

A Mechanism for Short-Term Associative Plasticity

It is likely that the mechanism of short-term associative plasticity is also important in other brain areas. Associative plasticity on this timescale has been proposed to underlie a network model of working memory (Sandberg et al., 2003). In addition, the strategy of making predictions before performing a task, generating error signals if there is deviation from the prediction, and then using error signals to improve performance is used in the cerebellum, striatum, superior colliculus, and cortex (Schultz and Dickinson, 2000). The endocannabinoid signaling system is also present in many brain regions where such predictive correction occurs (Egertova et al., 2003). In the cortex and hippocampus, endocannabinoids mediate long-term synaptic inhibition (Chevaleyre and Castillo, 2003; Sjostrom et al., 2003), and the possibility exists that endocannabinoid release could also be regulated by associative mechanisms and mediate short-term plasticity in these brain regions. Associative short-term plasticity could therefore regulate synaptic strength during tasks requiring dynamic regulation on rapid timescales, where an error signal drives endocannabinoid release.

Experimental Procedures

Electrophysiology

Parasagittal slices, 300 μm thick, were cut from the cerebellar vermis of 12- to 19-day-old Sprague-Dawley rats. Extracellular saline contained 125 mM NaCl, 26 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4-H_2O, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ and was bubbled with 95% O_2/5% CO_2. All procedures involving animals were approved by the Harvard Medical Area Standing Committee on Animals. BAPTA, fura-5F, and fura-FF were purchased from Molecular Probes (Eugene, OR). AM251 and CPA were purchased from Tocris Cookson (Ellisville, MO). All other chemicals were purchased from Sigma/RBI (St. Louis, MO)

Current-clamp experiments were performed using a Multiclamp 700A (Axon Instruments, Union City, CA). Glass electrodes (2–4 MΩ) were filled with an internal solution containing 130 mM $KMeSO_3$, 4.9 mM $MgCl_2$, 0.1 mM EGTA, 10 mM HEPES, 2 mM Na_2ATP, 0.4 mM NaGTP, 14 mM tris-creatine phosphate (pH 7.3; 310 mOsm). When BAPTA (20 mM) was used, it replaced $KMeSO_3$. In some experiments, BAPTA (20 mM) was added with 4 mM calcium to set resting calcium at ~60 nM. Free calcium for internal solutions containing BAPTA and calcium was calculated using the MaxChelator program (Stanford University) and verified by ratiometric fluorescence measurements using fura-5F. Experiments were performed at 33°C–34°C.

Postsynaptic Ca^{2+} Imaging

To measure postsynaptic calcium transients, the intracellular solution was supplemented with 500 μM fura-FF or 500 μM fura-5F. Imaging was carried out as previously described (Brenowitz and Regehr, 2003; Brown et al., 2003). Briefly, images were acquired at 50 Hz with 380 nm excitation, beginning 250 ms prior to the onset of conditioning trains. Images with excitation at the isosbestic point (F_{ISO}) of fura-FF (354 nm) or fura-5F (357 nm) were taken immediately before and after 380 nm excitation. Fluorescence ratios were converted to [Ca] using a value for the K_D of fura-FF of 7.7 μM and 400 nM for fura-5F (Brenowitz and Regehr, 2003; Grynkiewicz et al., 1985).

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